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Peer-reviewed

### Citation for published item:

Shuvaev, Sergey and Pal, Robert and Parker, David (2017) 'Selectively switching on europium emission in drug site one of human serum albumin.', *Chemical communications.*, 53 (50). pp. 6724-6727.

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<https://doi.org/10.1039/C7CC03071A>

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## Selectively switching on europium emission in drug site one of human serum albumin

Received 21st April 2017,  
Accepted xx May 2017

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DOI: 10.1039/x0xx00000x

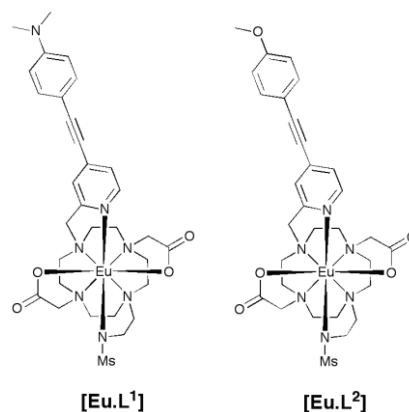
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**A luminescent europium probe has been discovered that binds selectively to drug-site I in human serum albumin, signalled by a 'switching on' of europium emission, and accompanied by strong induced circularly polarised luminescence.**

Albumin is the most abundant serum protein in mammals, and plays a key role in the transport both of endogenous compounds, such as fatty acids (FA) and hormones, and a wide range of drugs where it regulates their pharmacokinetics and bioavailability.<sup>1,2</sup> Detailed structural and computational studies have revealed its structural plasticity, with seven binding pockets present that explain its ability to bind many different types and sizes of compound.<sup>3-5</sup> The two main drug binding sites in human serum albumin (HSA) are termed Sudlow's drug site I and II, (DS-1, DS-2), and are distinguished by the fact that DS-1 binds predominantly by a solvophobic interaction, whereas DS-2 binding has been rationalised in terms of combined electrostatic, directed hydrogen-bonding and hydrophobic binding contributions, notably for anionic guests. The prototypical drugs that bind selectively to each of these sites in HSA are ibuprofen (DS-2; log K 6.4) and iodipamide (DS-1, log K = 7.0, 298 K, I = 0.1 M NaCl). These drugs bind with lower affinity to some of the other protein binding sites.<sup>1</sup>

There are significant structural differences in the albumins of different animal species. Bovine serum albumin (BSA), for example, has only 76% structural homology with HSA and the DS1 pocket is much more exposed to water, partly explaining the different affinity profile for a given drug or metabolite.<sup>6</sup> It also possesses an additional aromatic amino-acid residue, Trp-134, that is located in one of the weaker binding pockets, (FA-1). The importance of the serum albumins has triggered studies of their direct detection in serum or in urine, using

various luminescent probes.<sup>7-9</sup> The presence of HSA in urine signals the onset of renal malfunction, whilst a deficiency in blood is associated with liver disease, e.g. cirrhosis and chronic hepatitis.



We and others have been studying a range of long-lived lanthanide(III) probes, suitable for use in detecting changes in pH and pX (e.g. X = urate, citrate, lactate, bicarbonate) both *in vitro* and in living cells,<sup>10-14</sup> using spectral imaging in fluorescent confocal microscopy.<sup>15</sup> During the course of these studies, we evaluated the medium and pH-dependence of emission from the Eu(III) complexes, [Eu.L<sup>1</sup>] and [Eu.L<sup>2</sup>]. Aware of the ability of the conjugated chromophore to exhibit solvatochromism,<sup>16,17</sup> wherein the energy of the intramolecular charge transfer transition varies sensitively with solvent polarity, we have also examined their emissive behaviour in the presence of both BSA and HSA. Furthermore, this study has been extended to embrace live cell-imaging studies, changing the foetal calf-serum (containing BSA) in the cell-growth medium to goat and human serum, to assess the effect on emission and cell uptake.

Each of the ligands, L<sup>1</sup> and L<sup>2</sup>, was prepared from *trans*-1,7-bis(*tert*-butoxy-carbonylmethyl)-1,4,7,10

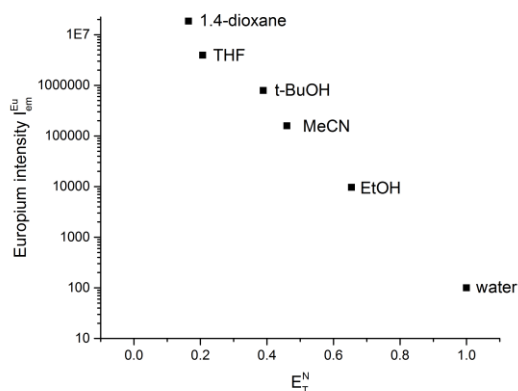
tetraazacyclododecane by stepwise alkylation reactions (ESI). The europium(III) complexes were purified by reverse-phase HPLC and exhibited significantly different photophysical

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Electronic Supplementary Information (ESI) available: [synthesis characterisation and series of additional pH and protein titrations are given in the supplementary information]. See DOI: 10.1039/x0xx00000x

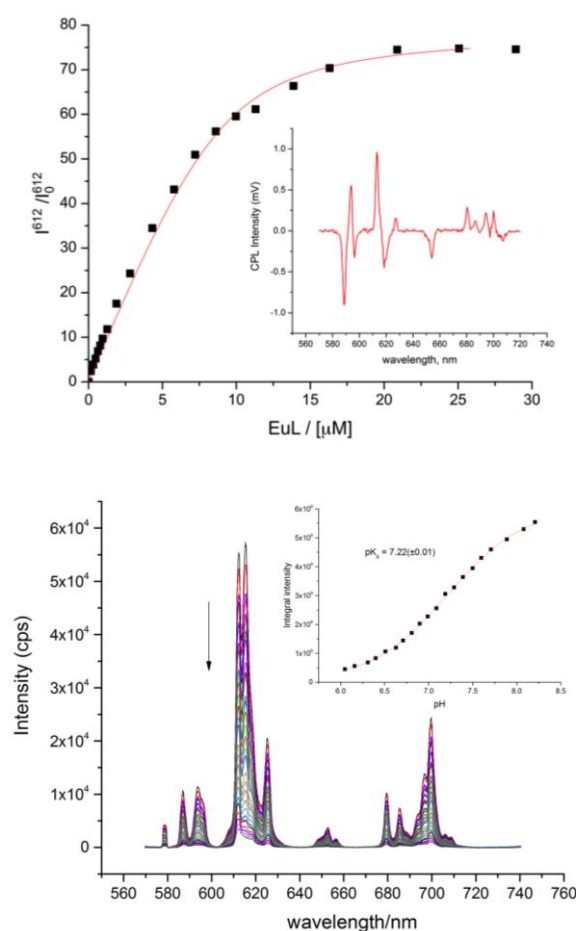
behaviour in solution, in accord with the sensitivity of the energy of the ICT excited state of [Eu.L<sup>1</sup>] to its local environment, (Table 1). The absorption spectrum of [Eu.L<sup>1</sup>] in water showed a very broad absorption band at 365 nm, but gave rise to no europium emission over the pH range 3 to 8. As the solvent polarity was reduced, the europium emission was 'switched on', being strongest in the least polar solvents, THF and 1,4-dioxane, (Figure 1).



**Figure 1** Variation of europium total emission with Reichardt's normalised solvent polarity parameter.

Moreover, incremental addition of HSA, to a solution of [Eu.L<sup>1</sup>] in phosphate-buffered saline (pH 7.4, 295 K) also led to a 'switching-on' of europium luminescence, (Figure 2) accompanied by a strong induced circularly polarised luminescence (CPL) signal, with  $g_{em}$  (589 nm) =  $-0.2$ . The emission intensity of the protein-bound complex was pH dependent, (Figure 2), with an apparent  $pK_a$  of  $7.22(\pm 0.01)$ , associated with reversible, intramolecular sulfonamide ligation. The form of the protein binding curve and the extended range of the sensitivity to added protein suggested binding of the complex may occur to more than one protein site. In the presence of a 100-fold excess of added HSA, the lifetime of europium emission was 0.3 ms, the intensity was insensitive to degassing the solution and the excitation spectrum showed a band at 390 nm, likely to be an internal charge transfer band. A significant bathochromic shift of this excitation band, when compared to the absorption spectrum of [Eu.L<sup>1</sup>] without serum albumin and its excitation spectra in different solvents (Figure 1) suggest the presence of intermolecular stacking interactions between alkynylpyridine moieties. Under the same conditions using added BSA, the lifetime of the observed emission was 0.18 ms, and the overall emission intensity was fifteen times lower, (Table 1). The emission behaviour is consistent with binding of the complex into a hydrophobic HSA site, e.g. DS1, that leads to a tightening of the ICT excited state manifold, and a more efficient overall europium sensitisation pathway. Accordingly, iodipamide – a drug that binds most strongly to DS-1, <sup>3</sup> was added incrementally to a solution containing [Eu.L<sup>1</sup>] (30  $\mu$ M) and HSA (9  $\mu$ M). The intensity dropped by a factor of five, and residual Eu luminescence was observed, even with a large

excess of added iodipamide, suggesting that [Eu.L<sup>1</sup>] was also bound to other hydrophobic binding sites on the protein. Competitive displacement of [Eu.L<sup>1</sup>] from DS-1 by iodipamide is occurring, (Figure 3), with an apparent affinity constant for [Eu.L<sup>1</sup>] binding of  $\log K = 5.29(06) M^{-1}$ , in reasonable agreement with the global value of 6.00, obtained by monitoring the 'switching on' of Eu emission by adding the free Eu complex to HSA (Figure 2). A parallel competitive binding experiment was undertaken with added ibuprofen, to probe affinity for drug-site II, (DS-2), (Figure 3). In this case, a two-step displacement curve was obtained, with a much smaller reduction of 50% in total emission intensity, suggesting that [Eu.L<sup>1</sup>] binds to DS-1 and DS-2 at least, with an overall site affinity that is of the order of  $3 \times 10^5 M^{-1}$ .



**Figure 2** (upper): variation of Eu emission intensity as [Eu.L<sup>1</sup>] is added to HSA (295K, 0.1 M NaCl, 11  $\mu$ M protein;  $\log K = 6.00(02)$ ; the inset shows the induced CPL spectrum recorded for the protein bound complex; (lower): the pH dependence of Eu emission in the human serum albumin bound complex (295K, 0.1 M NaCl, 10  $\mu$ M [Eu.L<sup>1</sup>]) fitted as a Boltzman sigmoid.

The affinity of the lanthanide(III) complex with BSA and HSA was also examined in a parallel study of the protein dependence of relaxivity changes with [Gd.L<sup>1</sup>].<sup>18</sup> Incremental addition of protein (BSA or HSA) to [Gd.L<sup>1</sup>] (1.2 mM, 310 K, 1.4 T) led to a 4-fold increase in the water proton relaxation rate

with HSA, compared to a 2.6 fold increase with BSA, (ESI). The similarity in the form of the binding curves suggested approximately equal *overall* protein affinities, with log K values of the order of 3.5 (0.2), (ESI). The pH dependence of the HSA/[GdL<sup>1</sup>] adduct revealed enhanced relaxivity at lower pH, as the sulphonamide nitrogen atom dissociated, with a pK<sub>a</sub> value of 6.36(06) in agreement with related protein-bound systems.<sup>19</sup> Addition of iodipamide or ibuprofen to each of the HSA-bound Gd complexes did not change the measured relaxivity by more than 10%, suggesting that the Gd complex also binds to protein binding pockets with moderate affinity that do not accommodate either of these drugs. The overall enhanced relaxivity value measured is caused by modulation of the rotational correlation time ( $\tau_c$ ) of the Gd complex and reflects the weighted contributions of the bound complex in the different binding sites.<sup>19</sup>

**Table 1.** Selected photophysical properties of [Eu.L<sup>1</sup>] and [Eu.L<sup>2</sup>] (295 K, H<sub>2</sub>O)

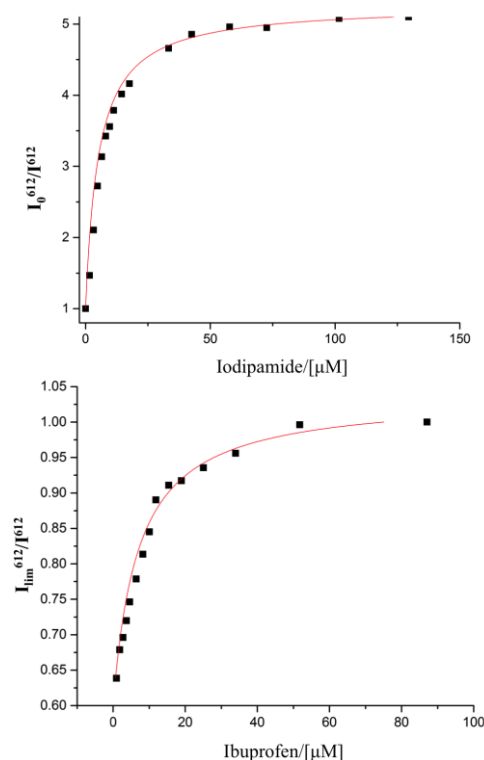
| Complex  | [Eu.L <sup>1</sup> ] | [Eu.L <sup>2</sup> ]   |
|--|----------------------|------------------------|
| $\lambda_{\text{abs}}/\text{nm}$                     | 365                  | 325                    |
| $\epsilon(\text{H}_2\text{O})$                       | 28,000               | 35,400                 |
| $\tau_{\text{H}_2\text{O}}/\text{ms}$                | c                    | 0.73 (pH 6)            |
| $\tau_{\text{D}_2\text{O}}/\text{ms}$                | c                    | 1.00 (pH 6)            |
| $\tau_{\text{H}_2\text{O}} + \text{HSA}/\text{ms}^a$ | 0.30                 | 0.79                   |
|  | 0.18                 | 1.02                   |
| $\phi_{\text{Eu}}/\%$                                | 0                    | 6.6                    |
| $\phi_{\text{Eu}} + \text{HSA}/\%^a$                 | 0.7                  | 6.0 (4.3) <sup>b</sup> |

<sup>a</sup> Added protein concentration was 0.7 mM; <sup>b</sup> value in parenthesis is for added BSA; <sup>c</sup> No measurable emission in solution.

The emission behaviour of [Eu.L<sup>2</sup>] was completely different to [Eu.L<sup>1</sup>]. Excitation into the primary absorption band at 325 nm led to long-lived europium emission that was not significantly changed in form or intensity in the presence of added HSA or BSA. The protein bound complex gave rise to a weak induced CPL signal, with  $g_{\text{em}}$  (589 nm) = −0.02. However, the form of the CPL spectrum was identical to that observed with [Eu.L<sup>1</sup>] suggesting a common local chiral protein environment, i.e. a common binding pocket in which Eu CPL can be observed. In saline solution, the pH-dependence of Eu emission was monitored; the spectral form, the emission intensity, and the lifetime increased with pH. An apparent pK<sub>a</sub> value of 3.80(06) was estimated (ESI) associated with reversible binding of the sulfonamide nitrogen atom to the Eu ion. This pK<sub>a</sub> value increased to 6.8 in a cell lysate medium.<sup>20</sup> In comparison, [Eu.L<sup>1</sup>] when bound to excess HSA also exhibited a pH-dependent emission response, with an apparent pK<sub>a</sub> around 7.22(05) (ESI). It had earlier been noted that in a hydrophobic binding pocket, e.g. DS-1, an apparent pK<sub>a</sub> may rise by up to 3 units versus bulk solution, i.e. the protonation equilibrium is significantly perturbed by protein association.<sup>21</sup>

Each Eu(III) complex was examined as a luminescent cellular stain in living mouse fibroblast cells (NIH-3T3) using both live cell epi and confocal microscopy. In the incubation medium, 10% foetal calf-serum (FCS) is normally added to promote cell growth; FCS contains BSA. Accordingly, different cell growth media were created to replace FCS based on added heat

inactivated purified culture grade human or goat serum. In the cell medium, the Eu emission could only be easily discerned by spectral imaging for the HSA-added preparation. Following an incubation of 4h in respective growth medium containing up to 100  $\mu\text{M}$  [Eu.L<sup>1</sup>] and keeping all experimental parameters constant, the Eu staining was 50% brighter for the HS-medium vs FCS-medium.

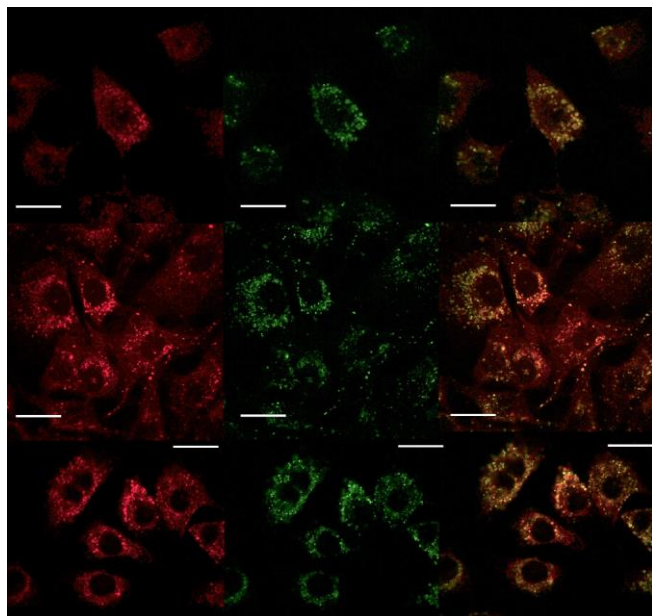


**Figure**

Variation of europium emission intensity in the presence of HSA showing the fit (line) to the data points: (upper): with added iodipamide ([Eu.L<sup>1</sup>] 30  $\mu\text{M}$ ; [HSA] 9.0  $\mu\text{M}$ ), log K = 5.29(03); (lower): with added ibuprofen ([Eu.L<sup>1</sup>] 26  $\mu\text{M}$ ; [HSA] 9.4  $\mu\text{M}$ , log K = 5.16(03), fitted assuming a 1:1 binding isotherm). Note that the y axis in the lower case, denotes  $I_{\text{final}}/I_0$ , at 612 nm, showing the 50% change.

A lysosomal staining pattern was confirmed by co-staining with LysoTracker Green<sup>TM</sup> ( $P > 0.83$ , ESI and Figure 4). The brightness of cell images was promoted to 120% increase vs FCS medium when the local lysosomal pH (normally around 4.2–4.5) was adjusted to 6.5, following addition of nigericin (5 min, 200 nM)<sup>10a</sup>. This study allowed consideration of the question as to whether it is enhanced intracellular uptake in the presence of HSA or enhanced complex brightness *in cellulo* that explains the variation of Eu emission intensity observed by microscopy. Independent measurement of the total Eu intracellular concentration for each incubation, was determined by ICP-MS measurements. No significant differences in Eu concentrations were observed between incubations of [Eu.L<sup>1</sup>] with added HSA and BSA (62% and 60% accumulation, respectively). However, the sample incubated with GSA showed a much lower intracellular concentration (29%). At the same time, [Eu.L<sup>2</sup>] incubated with added HSA

showed similar cellular uptake (55%), hinting that the mechanism of cellular uptake of [Eu.L<sup>1</sup>] and [Eu.L<sup>2</sup>] may be similar in nature.



**Figure 4** LSCM images (1024x1024 pixel, 100Hz bidirectional) in NIH-3T3 cells showing: (upper) predominantly lysosomal staining following a 4h incubation of 20  $\mu$ M complex ( $\lambda_{\text{ex}}$  = 355 nm,  $\lambda_{\text{em}}$  = 605–720 nm, 8 mW), and 5 min incubation of LTG ( $\lambda_{\text{ex}}$  = 488 nm,  $\lambda_{\text{em}}$  = 500–530 nm, 2 mW), (ambient lysosomal pH is ca. 4.5); (centre/lower): enhanced image intensity following a 5 min incubation with nigericin (200 nM) and LysoTracker Green (central columns), RGB merge highlighting correspondence (scale bar 20  $\mu$ M).

In summary, the luminescence of the europium complex, [Eu.L<sup>1</sup>] is switched on when bound to HSA but not to BSA or GSA. The europium complex prefers to bind to drug-site one and with relatively high affinity, suggesting potential uses in drug screening assays that probe the HSA protein site selectively. These observations have been extended to cell imaging studies. By replacing BSA (e.g. as calf serum) by HSA in the cell-growth medium, much more intense probe emission in the lysosomes could be observed, especially at the elevated pH values that are known to characterise the presence of lysosomal storage diseases.

**Acknowledgements** We thank Durham University for a doctoral studentship (SS) and EPSRC (EP/L019124/1) and the Royal Society (RP) for support. We would also like to express our gratitude to Dr. Chris Ottley for the ICP-MS measurements.

## References

- a) A. Bujacz, *Acta Cryst.* 2012, **D68**, 1278–1289; b) K. A. Majorek, P. J. Porebski, A. Dayal, M. D. Zimmerman, K. Jablonska, A. J. Stewart, M. Chruszcz and W. Minor, *Mol. Immunol.*, 2012, **52**, 174–182.
- J. Ghumain, P. A. Zunsain, I. Petitpas, A. A. Bhattacharya, M. Otagiri and S. Curry, *J. Mol. Biol.* 2005, **353**, 38–52.
- K. Yamasaki, V. T. G. Chuang, T. Maruyama, M. Otagiri, *Biochim. Biophys. Acta*, 2013, **1830**, 5435–5443.
- M. Fasano, S. Curry, E. Terreno, M. Galliano, G. Fanali, P. Narciso, S. Notari and P. Ascenzi, *Life*, 2005, **57**, 787–796.
- S. Evoli, D. L. Mobley, R. Guzzi, B. Rizutti, *Phys. Chem. Chem. Phys.* 2016, **18**, 32358–32368.
- S. Datta, M. Holder, *J. Phys. Chem. B*, 2014, **118**, 6071–6085.
- C. Liao, F. Li, S. Huang, B. Zheng, J. Du, D. Xiao, *Biosensors Bioelectronics*, 2016, **86**, 489–495.
- Y. Li, K. Li, J. He, *Luminescence*, 2016, **31**, 905–910.
- X. Wang, X. Wang, Y. Wang, Z. Guo, *Chem. Commun.* 2011, **47**, 8127–8129.
- a) D. G. Smith, B. K. McMahon, R. Pal, D. Parker, *Chem-Eur. J.* 2012, **18**, 11604–11613; b) Y. Bretonniere, M. J. Cann, D. Parker, R. Slater, *Org. Biomol. Chem.* 2004, **2**, 1624–1632.
- a) for an example of non-selective ibuprofen binding to BSA involving a europium labelled nanoparticle platform: S. Comby, T. Gunnlaugsson, *ACS Nano*, 2011, **5**, 7184–7197; b) for ratiometric analysis: R. A. Poole, F. Kielar, S. L. Richardson, P. A. Stenson, D. Parker, *Chem. Commun.* 2006, 4084–4086.
- R. Pal, A. Beeby, D. Parker, *J. Pharm. Biomed. Anal.* 2011, **56**, 352–358.
- B. K. McMahon, D. Parker, *RSC Adv.* 2014, **4**, 37649–37654.
- S. J. Butler, M. Delbianco, L. Lamarque, B. K. McMahon, E. R. Neil, R. Pal, D. Parker, J. W. Walton, J. M. Zwieter, *Dalton Trans.* 2015, **44**, 4791–4803.
- R. Pal, *Faraday Trans.* 2015, **177**, 507–515.
- a) W.-S. Lo, J. Zhang, W.-T. Wong and G.-L. Law, *Inorg Chem.*, 2015, **54**, 3725–3727; b) W.-S. Lo, W.-T. Wong and G.-L. Law, *RSC Advances*, 2016, **6**, 74100–74109.
- a) M. Soulie, F. Latzko, E. Bourrier, F. Placide, S. J. Butler, R. Pal, J. W. Walton, P. L. Baldeck, B. Le Guennic, C. Andraud, J. M. Zwieter, L. Lamarque, D. Parker, O. Maury, *Chem-Eur. J.* 2014, **20**, 8636–8646; b) A. Picot, F. Malvotti, B. Le Guennic, P. L. Baldeck, J. A. G. Williams, C. Andraud, O. Maury, *Inorg. Chem.* 2007, **46**, 2659–2665; b) A. D'Aleo, A. Picot, A. Beeby, J. A. G. Williams, B. Le Guennic, C. Andraud, O. Maury, *Inorg. Chem.* 2008, **47**, 10258–10268.
- a) P. Caravan, *Acc. Chem. Res.*, 2009, **42**, 851–862; b) H. B. Eldredge, M. Spiller, J. M. Chasse, M. T. Greenwood, P. Caravan, *Invest. Radiol.* 2006, **41**, 229–243.
- a) L. Moriggi, M. A. Yaseen, L. Helm, P. Caravan, *Chem-Eur. J.* 2012, **18**, 3675–3686; b) M. P. Lowe, O. Reany, S. Aime, M. Botta, G. Castellano, E. Gianolio, D. Parker, *J. Am. Chem. Soc.*, 2001 **123**, 7601–7609.
- B. K. McMahon, R. Pal and D. Parker, *Chem. Commun.* 2013, **49**, 5363–5365.
- A. V. Onufriev and E. Alexov, *Q. Rev. Biophys.* 2013, **46**, 181–209.